ENANTIOSELECTIVE ESTERIFICATION OF IBUPROFEN USING LIPASE IMMOBILIZED ON VARIOUS SUPPORTS IN COMPARISON WITH FREE LIPASE

Dissertation submitted to

THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI

Submitted by

NANCY RITA.M

Under the guidance of

Dr. S. KRISHNAN, M.Pharm., Ph.D.

Prof &Head, Department of Pharmaceutical Biotechnology

in partial fulfilment for the award of degree of

MASTER OF PHARMACY

(Pharmaceutical Biotechnology)

MARCH 2009

COLLEGE OF PHARMACY

SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES

Coimbatore – 641044

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Certificate

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was carried out by

NANCY RITA.M

in the Department of Pharmaceutical Biotechnology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai, under my direct supervision and guidance to my fullest satisfaction.

Place: Coimbatore

Date:

Dr. S. KRISHNAN, M.Pharm., Ph.D.

Prof & Head

Department of Pharm. Biotechnology,

College of Pharmacy, SRIPMS,

Coimbatore.

Certificate

This is to certify that the analytical part of the research work entitled

ENANTIOSELECTIVE ESTERIFICATION OF IBUPROFEN USING LIPASE IMMOBILIZED ON VARIOUS SUPPORTS IN COMPARISON WITH FREE LIPASE

was carried out by

NANCY RITA.M

in the Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai.

Place: Coimbatore
Date:

Dr.T. K. Ravi, M.Pharm., Ph.D.,FAGE.,
Head - Dept. of Pharmaceutical Analysis,
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ACKNOWLEDGEMENT

In my opinion, doing research is like running an off-road track. One can know in which direction to go, however, one cannot foresee what will be coming along the way. The varying nature of challenges one meets stimulate one's mind and the passage of each obstacle gives a feeling of satisfaction and adds to the excitement of what is to come. Now, close to the finishing line of this run, I would like to address my sincere gratitude to the persons who have accompanied me along the course and those who have been there by the side to support me.

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Above all I am grateful to my parents and my beloved ones for their endless support and love during this work as in all stages of my life.

Nancy Rita. M.

PURPOSE OF WORK

Since 1985, there has been an increasing trend to produce and market chiral drugs composed of single active enantiomers rather than as racemates, which contain both active and inactive isomers. Due to this resolution, there is now an absolute need to produce new drugs with a high optical purity or old drugs are enantio-enriched so that they show a high optical purity.

Over the last decade, an increasing number of studies utilizing enzymatic biotransformations have been reported. Lipase is the biocatalyst most frequently used in organic synthesis, particularly because lipase has an enantioselective reaction property for racemic mixtures (Muralidhar *et al* 2001).

Ibuprofen is an important member of the non-steroidal, anti-inflammatory drugs (NSAID'S) consisting of the 2-arylpropionic acids (profens). The racemic mixture of its two enantiomers S- (+) and R- (-) is being used, but its biological activity resides mainly with the S-enantiomer. Studies project that over the next few years, (S)–ibuprofen has the potential of becoming a one billion dollar per year over the counter medication (Trani M *et al* 1998).

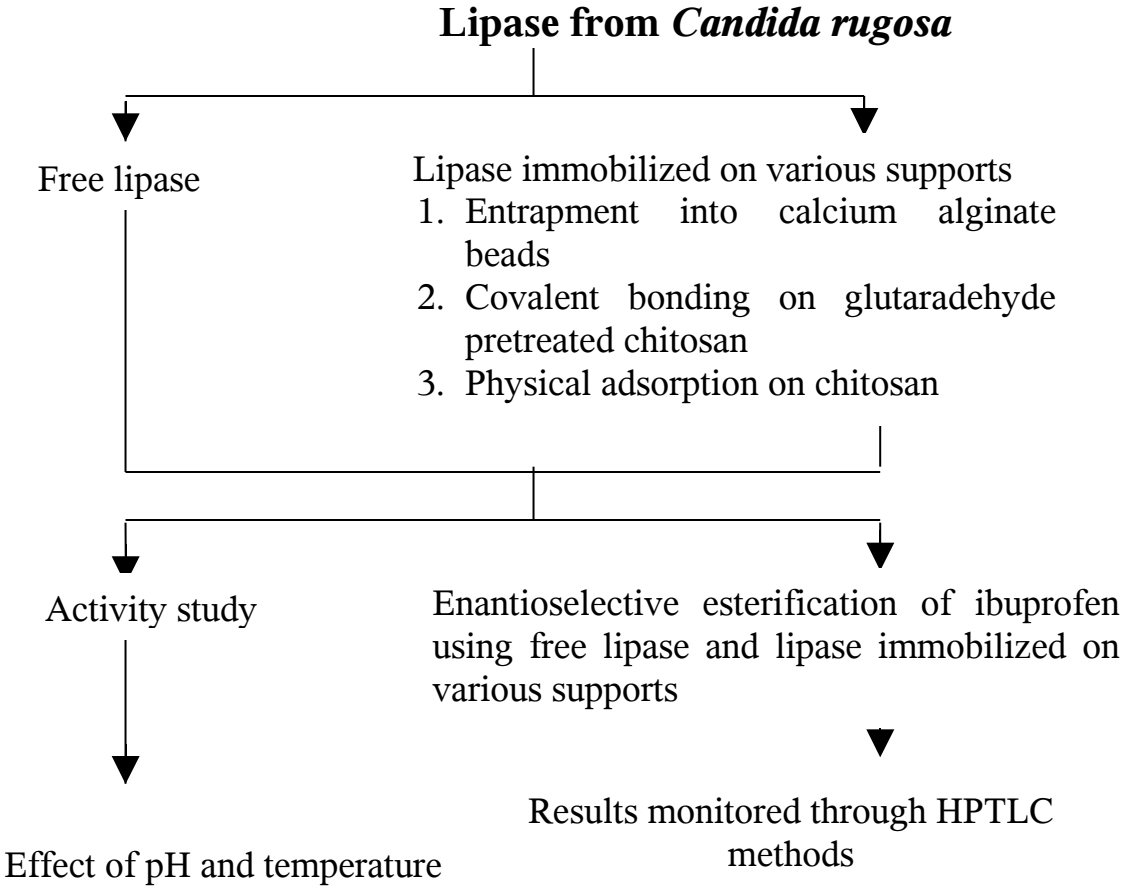
Previous work carried out by Yu. H *et al* in 2004 proved that the enantioselectivity of *Candida rugosa* lipase increased 2.2 times when it was immobilized, for ibuprofen resolution. Works like resolution of racemic ibuprofen by Mustranta in 1992 and enzymatic synthesis of (S) - ibuprofen ester from racemic ibuprofen using lipases in organic solvent by Tsai SW *et al* 1997 inspired us to carry out the work on enantioselective esterification of ibuprofen using immobilized lipase.

Lipase immobilized on various supports by different techniques were applied in oil hydrolysis by Vimla Minovska *et al* in 2005. Among this Amberlite IRC- 50 was stable when compared with other supports.

Each support has advantages of its own. Yuko Ikeda *et al* in 2002 used cellulose acetate titanium isopropoxide gel fiber as support for lipase immobilization which retained enantioselectivity at 30^{o}C.

The present work focuses on activity studies of free lipase and lipase immobilized on various supports and to compare the effect of immobilized lipase and free lipase on enantioselective esterification of ibuprofen.

SCHEME OF WORK



ABSTRACT OF WORK

- Immobilization of *Candida rugosa* lipase on various supports.

    1. Entrapment into calcium alginate beads
    2. Covalent bonding onto glutaraldehyde pretreated chitosan
    3. Physical adsorption on chitosan

- Activity studies of free and immobilized *Candida rugosa* lipase.

- Enantioselective esterification of ibuprofen using free and immobilized lipase from *Candida rugosa* lipase was monitored by HPTLC method.

- The efficiency and enantioselectivity of free and immobilized lipase was compared.

INTRODUCTION

The steadily growing interest in lipases over the last two decades stems from their biotechnological versatility and the ability of these enzymes to catalyze a broad spectrum of bioconversion reactions with tremendous potential in various areas such as food technology, biomedical sciences and chemical industry. Many of these applications are performed with immobilized lipases. Immobilization is an advantageous method that improves the stability of the biocatalyst and provides for its repeated use and easy separation of catalyst from the reaction medium. Various techniques using varied support materials have been studied and consequently many immobilized preparations with a wide range of efficiency, stability and activity are available.

Lipases have been covalently bound to activated poly (vinyl chloride), nylon, Eupergit or to controlled pore silica. Non-covalently attached or entrapped enzymes have been prepared in polyionic chitosan hydrogel and alginate gels. Adsorption on hydrophobic or hydrophilic supports, as a simple method, still attracts attention. Among the various supports, celite, cellulose, ethyl cellulose, carbon and synthetic polymers, as well as rice straw and alumina beads, have served as carriers for this purpose. Recently, a novel form of lipase covalently immobilized on reversibly soluble polymers was proposed. Another novel technique for the immobilization of lipase on colloidal gas aphrons on spherical microbubbles was studied by **Vilma Minovska *et al*., 2004.**

LIPASES

Lipases as triacylglycerol ester hydrolases, (EC. 3.1.1.3) are ubiquitous enzymes that catalyse the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. These enzymes are distributed among higher animals, microorganisms and plants in which they fulfil a key role in the biological turnover of lipids.

They are required as digestive enzymes to facilitate, not only the transfer of lipid from one organism to another, but also the deposition and the mobilization of fat that is used as an energy reservoir within the organism. They are also involved in the metabolism of intracellular lipids, and, therefore, in the functioning of biological membranes. Lipases have been extensively investigated with respect to their biochemical and physiological properties, and lately for their industrial applications. The increasing interest in lipase research over the past decades has likely occurred for three reasons. The first is related to the molecular basis of the enzyme catalytic function. The second reason is linked to the enzyme's medical relevance and its importance in regulation and metabolism. Lastly, it was discovered that lipases are powerful tools for catalysing not only hydrolysis, but also various reverse reactions, such as esterification, transesterification and aminolysis in organic solvents. The possible reaction ways are presented in figure 1. Such biocatalysts present some important advantages over classical catalysts. Indeed, their specificity, regioselectivity and enantioselectivity allow them to catalyse reactions with reduced side

products, lowered waste treatment costs and under conditions of mild temperature and pressure. Accordingly, considerable attention has been given lately to the commercial use of lipases.

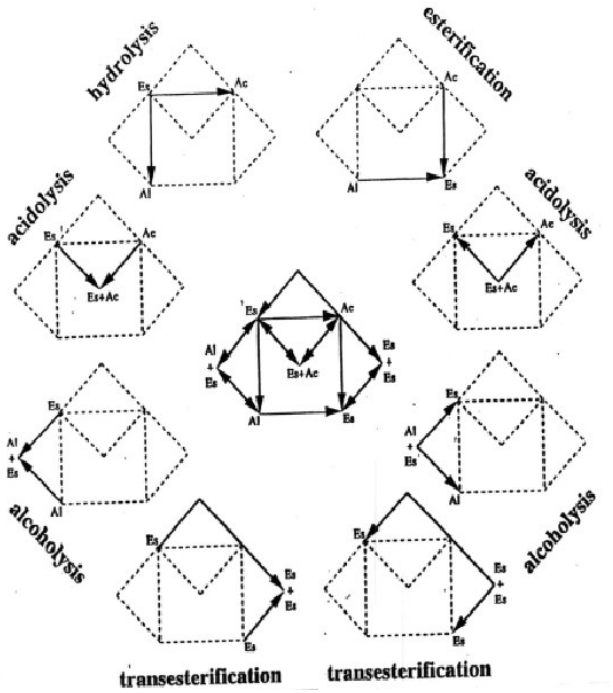


Figure 1: Possible reaction catalysed by lipase enzyme reaction

Because of their capability to preserve their catalytic activity in organic solvents, the activities of lipases as catalysts have been investigated to determine their potential for the conversion of surplus fats and oils into higher value products for food and industrial uses. Further examples of their applications are numerous and are found in the resolution of racemic mixtures, the synthesis of pharmaceuticals and new surfactants, the bioconversion of oils, fats, etc. However, the low stability, low activity or selectivity encountered occasionally with a number of these enzymes, and the relatively prohibitive cost of native enzyme have been the chief obstacle hindering more rapid expansion of industrial lipase technology on a large scale. Therefore, customisation of lipases by chemical and physical modifications has more recently been attempted to improve their catalytic properties in hydrolysis and synthesis involving aqueous and non-aqueous solvents **(Amir Kabiri Badr, 2005).**

IMMOBILIZATION

Enzymes have been used for several years to modify the structure and composition of foods but they have only recently become available for large-scale use in industry, mainly because of the high cost of enzymes. However, progress in genetics and in process technology may now enable the enzyme industry to offer products with improved properties and at reduced costs. Economical usage of lipases in industry requires enzyme immobilization, which enables enzyme reuse and facilitation of the continuous process. Immobilized enzymes are defined by Katchalski-Katzir at the first Enzyme Engineering Conference, held at Henniker, NH, USA, in 1971, as the confinement or localization of enzyme physically in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously. The concept of immobilizing proteins and enzymes to insoluble supports has been the subject of considerable research for over 30 years and consequently, many different methodologies and a vast range of applications have been suggested. Aims often include such factors as the reuse or better use of enzymes, especially if they are scarce or expensive, better quality products as there should be little enzyme in the product requiring inactivation or downstream purification, the production of biosensors, flow-through analytical devices or the development of continuous manufacturing processes. Although large tonnages of immobilized enzymes are used industrially, for example in the production of various syrups from starch, and there are several smaller-scale industrial applications, the introduction of such biocatalysts has been disappointingly slow. With many current manufacturing applications the cost of the enzyme itself is not a large proportion of overall production costs but the trend toward more complex processing operations and more sophisticated products in the pharmaceutical, food, chemical and other bioprocessing industries will require the use of a wider range of enzymes of greater purity and specificity and hence, higher value. Optimizing use and reuse of enzymes will be increasingly required.

Nelson and Griffin carried out the first investigation on immobilization in 1916.

In their work, invertase was adsorbed both on charcoal and aluminium hydroxide. Meanwhile, the first lipase immobilization study was carried out in 1956 by Brandenberger H. who covalently linked the lipase on ion-exchange resin. However, the first attempts for the usage of immobilized lipase for hydrolysis or ester synthesis reactions were done by Iwai et al. in 1964. The first industrial application of immobilized enzymes was reported by Tosa et al. 1967 at the Tanabe Seiyaku in Japan who developed columns of immobilized *Aspergillus oryzae* aminoacylase for the resolution of synthetic, racemic -amino acids into the corresponding, optically active enantiomers. Around 1970, two other immobilized systems were launched on a pilot-plant scale. In England, immobilized penicillin acylase, also referred to as penicillin amidase, was used to prepare 6-amino penicillanic acid (6-APA) from penicillin G or V, and in the USA, immobilized glucose isomerase was employed to convert glucose into fructose. These successful industrial applications prompted extensive research in enzyme technology, leading to a steady increase in the number of industrial processes based on sophisticated, immobilized- enzyme reactors.

Advantages of Immobilization

Immobilization process seems to offer mainly the economical advantages. However, there are a number of advantages to attaching enzymes to a solid support and a few of the major reasons are listed below:

- Enzymes can be reused.
- Processes can be operated continuously and can be readily controlled.
- Products are easily separated.
- Effluent problems and materials handling are minimized.
- In some cases, enzyme properties (activity and stability) can be altered favorably by immobilization.
- Provides higher purity and product yields, product inhibition is less apparent.
- Greater pH and thermal stability.

- No contamination due to added enzyme.
- Continuous operation.
- Greater flexibility in reactor design.

Factors Affecting Immobilization Performance

There are many factors that influence the performance of an immobilized enzyme preparation. Some of the most important factors are the choice of a carrier and the selection of an immobilization strategy.

Support Materials

An enormous number of different types of matrices have been used in laboratory immobilization studies. However, selection of the optimum support is the major parameter that affects the immobilization performance. The properties that an enzyme carrier should have can be listed as follows:

- Large surface area
- Permeability
- Insolubility
- Chemical, mechanical and thermal stability
- High rigidity
- Suitable shape and particle size
- Resistance to microbial attach
- Regenerability

However, the exact nature of the process design, the physical properties of the feedstock and the product, the reaction conditions and many other factors will place constraints on the type of matrix, which will be most suitable. In an industrial operation, maximized enzyme-matrix life span is vital component.

IMMOBILIZATION TECHNIQUES

The selection of an immobilization technique is based on process specifications for the catalyst, including parameters such as overall enzymatic activity, effectiveness of the lipase utilization, deactivation and regeneration characteristics, cost of immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase. Chemical methods feature the formation of covalent bonds between the lipase and the modifier, while physical methods are characterized by weaker interactions of the enzyme with the support material, or mechanical containment of the lipase within the support.

Methods for enzyme immobilization can be classified into three main categories**:**

- Carrier binding
- Cross linking
- Entrapment

Carrier Binding

Carrier-Binding method is the oldest immobilization method for enzymes and is defined as the binding of enzymes to water-insoluble carriers. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depends on the nature of the carrier. The following picture shows how the enzyme is bonded to the carrier in this method**:**

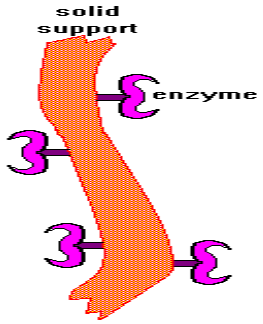
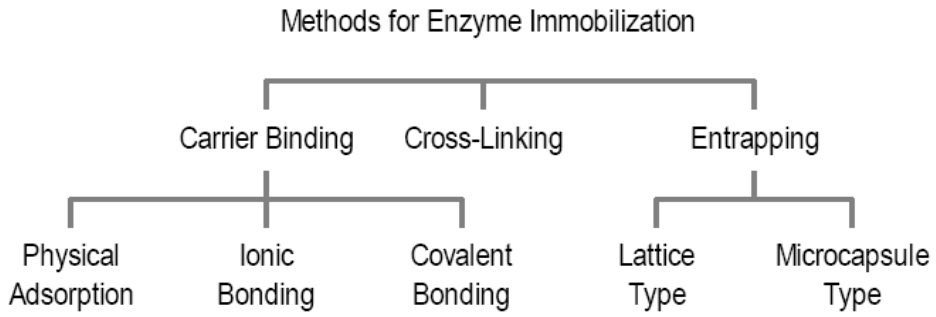


Figure 2: Schematic illustration of carrier-binding method

(http://www.eng.rpi.edu/dept/chem-eng/Biotech Environ/IMMOB/immob.htm).

Table 1: Enzyme immobilization methods



The selection of the carrier depends on the nature of the enzyme itself, as well as the:

- Particle size
- Surface area
- Molar ratio of hydrophilic to hydrophobic groups
- Chemical composition.

In general, an increase in the ratio of hydrophilic groups and the concentration of bounded enzyme, results in a higher activity of the immobilized enzymes. The most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel. According to the binding mode of the enzyme, carrier-binding method can further be sub-classified into:

1. Physical Adsorption
2. Ionic Binding
3. Covalent Binding

Physical Adsorption

The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-furanosidase onto aluminium hydroxide. This method for

the immobilization of enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme, or destruction of its active center. If a suitable carrier is found, this method can be both simple and cheap. They lend themselves a minimal resistance in the reaction mixtures, and possible supports for physical adsorption are mechanically durable and re-useable. Thus, this method is found to be the most suitable for large-scale immobilization.

However the disadvantage is that, the adsorbed enzyme may leak from the carrier during utilization, because the binding force between the enzyme and the carrier is weak. The enzyme is immobilized onto a solid support by low energy binding forces, e.g., Van der Walls interactions, hydrophobic interactions, hydrogen bonds, ionic bonds. Many carrier materials exist, the choice of one often depending on properties that are important for potential industrial applications mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme load capacity and cost. Initially, mineral supports such as porous glass beads, diatomaceous earth, silica and alumina were used. More recently, the most used supports are ion exchange resins, celite and biopolymers.

The success and efficiency of the physical adsorption of the enzyme on a solid support is dependent of several parameters. The size of the protein to be adsorbed, the specific area of the carrier and the nature of its surface (porosity, pore size) are crucial.

Typically, the use of a porous support is advantageous since the enzyme will be adsorbed at the outer surface of the material and within the pores as well. An efficient immobilization is also dependent on the enzyme concentration. The amount of adsorbed enzyme per amount of support increases with the enzyme concentration reaching a plateau at the saturation of the carrier. This operation is usually carried out at constant temperature, and consequently, adsorption isotherms are obtained which follow the

Langmuir or Freundlich equations. The pH at which the adsorption is conducted is equally important since ionic interactions are crucial in such an immobilization. Usually, the maximum adsorption is observed for pH values close to the isoelectric point of the enzyme. Finally, addition of water miscible solvents during the immobilization process favors the adsorption by reducing the solubility of the enzyme in the aqueous phase.

Lipase immobilization by physical adsorption finds application in bioconversion of oils and fats. Their unique specificities allow the design of synthetic routes that predetermine product structure and distribution whereas chemical catalysts generally lead to random reaction product mixtures. Thus, the use of lipases makes it feasible to obtain new products with predetermined physical and chemical properties.

During the immobilization of lipase by physical adsorption, driving force is mainly hydrophobic interaction because of the structural properties of the enzyme. Hydrophobic interaction arises from the repulsion of polar and non-polar molecules. Hydrophobic interactions are not a binding of hydrophobic groups to each other, these interactions are forced on the non-polar compounds by the polar environment such as water. It is the structure of the water that creates hydrophobic interactions. If the structure of the water changes by dissolving salts or organic solvents in water, then hydrophobic interactions would be affected. Generally, increasing ionic strength increases hydrophobic interactions. In addition to ionic strength, temperature and pH also affects the strength of interaction.

Ionic Binding

This method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Polysaccharides and synthetic polymers having ion-exchange centers are usually used for carriers. The binding of enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the

conformation and the active site of the enzyme, and so yields immobilized enzymes with high activity in most cases. As the binding forces between enzyme protein and carriers are less strong than in covalent binding, leakage of enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH.

The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkage is much stronger for ionic binding although less strong than in covalent binding.

Covalent Binding

The covalent binding method is based on the binding of enzymes and water insoluble carriers by covalent bonds. The functional groups that take part in this binding of enzyme to carrier can be amino, carboxyl, sulfhydryl, hydroxyl, imidazole or phenolic groups which are not essential for the catalytic activity. In order to protect the active site, immobilization can be carried out in the presence of its substrate or a competitive inhibitor. Activity of the covalent bonded enzyme depends on the size and shape of carrier material, nature of the coupling method, composition of the carrier material and specific conditions during coupling.

The main advantage of the covalent attachment is that such an immobilization is very solid. Unlike physical adsorption, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength. Moreover, the obtained immobilized enzymes are usually very stable and resistant to extreme conditions (pH range, temperature). Finally, a large number of different supports and methods to activate them are available. However, experimental procedures are obviously more difficult to carry out than for physical adsorption. The 3-D structure of the protein is considerably modified after the

attachment to the support. This modification generally leads to a significant loss of the initial activity of the biocatalyst.

Examples of derivatized lipases obtained through this procedure include PEG modified lipase, fatty acid-modified lipase, amidinated lipase, and detergent modified lipase.

Cross-Linking

It can be defined as the intermolecular cross-linking of enzymes by bifunctional or multifunctional reagents and it is based on the formation of chemical bonds, as in covalent binding method, but water-insoluble carriers are not used. The most common reagent used for cross-linking is glutaraldehyde. Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.

This method can be carried out in three different ways:

- Mixing the prepolymers with a photosensitizer (e.g., benzoin ethyl ether), melting, mixing with an enzyme solution and gelling by exposure to near ultraviolet radiation.
- Freezing a monomer solution containing the enzyme in the form of small beads. Polymerization is then started using gamma radiation.
- Mixing the enzyme in a buffered aqueous solution of acrylamide monomer and a cross-linking agent. Polymerization can be initiated by the addition of some chemicals.

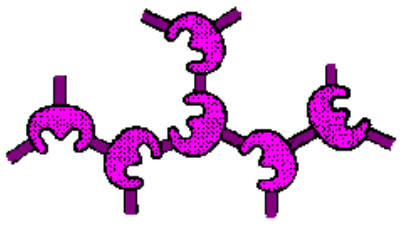
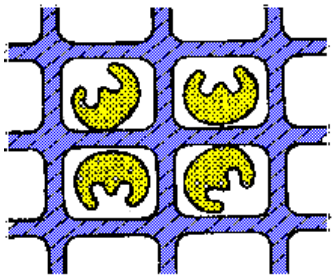


Figure 3: Schematic illustration of cross-linking method

(http://www.eng.rpi.edu/dept/chem-eng/Biotech-Environ/IMMOB/immob.htm).

Entrapment

Incorporating enzymes into the lattices of a semipermeable gel or enclosing the enzymes in a semipermeable polymer membrane. The entrapment for immobilization is based the localization of an enzyme within the lattice of a polymer matrix or membrane in such a way as to retain protein while allowing penetration of substrate. This method differs from the covalent binding and cross-linking in that enzyme itself does not bind to the gel matrix or membrane; and thus, has a wide applicability. The conditions, used in the chemical polymerization reaction, are relatively severe, resulting in the loss of enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required.



entrapped in a matrix

entrapped in droplets

ethods

MOB/immob.htm).

Entrapment can be classified into lattice and microcapsule types.

- Lattice-Type entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as polyacrylamide, polyvinylalcohol, etc. and natural polymer (starch) have been used to immobilized enzymes using this technique.

- Microcapsule-Type entrapping involves enclosing the enzymes within

semipermeable polymer membranes. This is probably the less developed immobilization technique, is very similar to entrapment, although in this case, it is the enzyme and its whole environment that are immobilized. Microencapsulation creates artificial cells delimited by a membrane. Large molecules such as enzymes are not able to diffuse across the synthetic membrane whereas small molecules, e.g., substrates and products, can pass through it (Villeneuve et al., 2000). The preparation of enzyme microcapsules requires extremely well controlled conditions; and the procedures for microcapsulation of enzymes are liquid drying, phase separation and interfacial polymerization method.

The advantage of such an immobilization technique is that the enzyme does not chemically interact with the polymer; therefore, denaturation is usually avoided. However, mass transfer phenomena around the membrane are problematic. The diffusion rate across the membrane of the substrate and the product is often the limiting parameter. Generally, high substrate concentrations are necessary in order to limit its influence. Finally, entrapped enzymes are better used with small substrates since larger ones may not be able to pass the membrane and reach the active site of the biocatalyst **(Banu, 2001).**

LIPASE IMMOBILIZATION

Immobilization of Lipase on Chitosan

Lipase was immobilized by physical adsorption on chitosan following a previously developed methodology with slight modifications. Chitosan *in natura* (2 g) had been previously soaked in hexane under agitation conditions (100 rpm) for 1 hour. Then, excess hexane was removed followed by the addition of 0.5 grams of powder lipase dissolved in 10 mL of distilled water. The lipase was on the support under agitation for 3 hours at room temperature followed by an additional period of 18 hours under static conditions at 4º C. The derivative was filtered (Whatman filter paper 41)

and thoroughly rinsed with hexane **(E.B.Pereira *et al* 2003).**

Immobilization of lipase on glutaraldehyde pre-treated chitosan

Immobilization of enzymes on glutaraldehyde-pretreated supports has been described in literature. In particular, immobilization of invertase on support materials activated with the dialdehyde has received great attention. In reference to the support material studied in this contribution, glutaraldehyde has been used by a number of authors as a coupling agent in the immobilization of enzymes onto chitosan powders. The dialdehyde has also been frequently used to introduce intermolecular crosslinking in proteins or to modify adsorbed proteins on aminated supports. The exact structure of glutaraldehyde on the support is under discussion, but polymers, monomers and dimers of glutaraldehyde onto different surfaces have been proposed to be present. The control of support activation with glutaraldehyde is very important. It has been reported that monomers and dimers of glutaraldehyde have different reactivity: while the dimer is able to rapidly immobilize proteins via a direct covalent attachment, the monomer yields a very low immobilization rate. Activity/stability properties of enzymes immobilized on glutaraldehyde activated supports depend on the exact immobilization protocol employed. Due to the existence of one or two ionic groups (amino groups) under the glutaraldehyde, which provide a certain anionic exchanger nature to the support, altering the ionic strength during the immobilization can modify the immobilization rate and also the region of the protein that is implied in the interaction with the support. Since in this contribution immobilization was performed in neutral medium, reaction should have involved the most reactive amino groups in the protein.

i) **Preparation of glutaraldehyde-pretreated chitosans**

Glutaraldehyde-modified supports were prepared by suspending 375mg of chitosan powder in 50 ml of 0.025% or 0.25% (v/v) glutaraldehyde/phosphate buffer pH 7.0 solutions. The suspension was kept under 800rpm stirring at room temperature for

30 min. After that time the supports were recovered by filtration and washed with distilled water. The modified supports (CHIT/GLU 0.025 and CHIT/GLU 0.25) were stored at 50 ^{0}C for 12 hrs.

ii) Lipase immobilization

The immobilization of lipases was performed at room temperature during 7 h with 350 rpm stirring. Phosphate buffer kept pH at 7, and the ionic strength of the immobilization medium equal to 0.014 M. In the case of CRL (solid powder), 150 mg of these lipases were added to 50 ml of phosphate buffer solutions, and subjected to strong stirring during 30 min in order to solubilize lipase. A filtering step was then performed in order to retain carbohydrates and other insoluble compounds. In the case of CALB (liquid ambar solution), 0.9 ml of the enzyme commercial solution were diluted up to 50 ml with phosphate buffer. Each lipase solution was then contacted with 375 mg of chitosan powder and glutaraldehyde-pretreated chitosans (room temperature, 350 rpm). After the desired contact time (7–8 h) solids were recovered by filtration, washed with distilled water and dried at 50 ^{0}C for 12 hrs **(M. L. Foresti and M.L. Ferreira in 2006).**

Immobilization of lipase by gel entrapment

Natural polysaccharides (e.g. agar, alginates, κ-carrageenan), gel-forming proteins (e.g. gelatin) and synthetic polymers (e.g. polyacrylamide) have gained a leading role as cell carriers in entrapment or encapsulation techniques. Among these, spherical matrices based on calcium alginate gels are the most widely used supports for immobilization of living cells. Alginates are naturally derived linear copolymers of 1, 4-linked β-D-mannuronic acid and α-L-guluronic acid residues. The ratio and sequential distribution of β-D-mannuronic acid (M) residues and α-Lguluronic acid (G) residues along the length of the alginate chain vary in alginates of different origins (brown seaweeds, certain bacteria). There is no regular repeat unit in alginate polymers, and the chains can be described as a varying sequence of regions termed M blocks, G blocks, and MG

blocks. Water solutions of polysaccharides form hydrogels in the presence of divalent ions via ionic interactions between acid groups on G blocks and the gelating ions, generally Ca^{2+}.

The lipase powder was dissolved in Tris–HCl buffer (50 mM, pH 7.3) to obtain a 4 % w/v solution. This solution was added to sodium alginate to yield either a 1.5 % w/v or a 2.5 % w/v mixture which was left to deaerate over night at 25 °C. By dropping the lipase–alginate mixture through a syringe into an aqueous solution of $CaCl_2$ (0.1 M), gel beads of about 3-mm diameter were formed within 3 to 5 min. The beads were kept in $CaCl_2$ solution until used.

LITERATURE REVIEW

- **Maha Karra-Chaabouni *et al.*, in 2008** reported the immobilization of *Rhizopus oryzae* lipase onto cellulose substrate. He studied the effect of temperature, pH and solvent polarity on the immobilized *Rhizopus oryzae* lipase and found that immobilized enzyme can be reused three times without significant loss of the catalytic activity.

- **Waze Aimee Mireille Alloue *et al.*, in 2008** immobilized lipase isolated from *Yarrowia lipolytica* using three methods including inclusion, adsorption, and covalent bond to study enzyme leaching, storage, and catalytic properties. He reported that adsorption and covalent bond allow multiple reuses of immobilized enzymes.

- Optical resolution of (R, S)-ibuprofen in organic solvent by porcine pancreatic lipase catalyzed enantioselective esterification was reported by **Ganesh Ramachandran and Laxmi Ananthanarayan in 2008.** They concluded that when surfactant coated porcine pancreatic lipase was used, the esterification reaction increased from 21 to 22.5%.

- **Gottemukkala VV *et al.,* in 2008** had reported effect of methyl branching of C_8H_{18} alkanes and water activity on lipase-catalyzed enantioselective esterification of ibuprofen. A minimal quantity of water was required for an enzyme to perform its function in organic media and an increase in methyl branching allowed higher enantioselectivity without necessarily increasing the initial reaction rate.

- **Mohd. Basyaruddin Abdul Rahman *et al.*, in 2008** studied the synthesis of methyl adipate ester from *Candida rugosa* lipase immobilized on Mg, Zn and Ni of

layered double hydroxides (LDHs). Modified advanced material of layered double hydroxides of Mg/Al, Zn/Al and Ni/Al showed to be well-suited support for immobilization of lipase for esterification in hexane

- **Roila Awang *et al.*, in 2007** immobilized lipase from *Candida rugosa* onto palm-based polyurethane foam through the physical adsorption method.

- **Wang Y *et al.,* in 2007** have studied the chiral separation of racemic ibuprofen. In this work, a special microstructure in the composite hydrophilic cellulose acetate (CA)/hydrophobic polite trafluoroethylene (PTFE) membrane was designed for lipase immobilization by ultrafiltration. A biphasic enzymatic membrane reactor (EMR) and an emulsion reaction system with free lipase were both used, and the activity, enantioselectivity and half-life of immobilized and free enzymes were compared.

- **Carvalho PO *et al.,* in 2006** had reported enzymatic resolution of (R, S)-ibuprofen and (R, S)-ketoprofen by microbial lipases from native and commercial sources. A preliminary optimization with respect to the influence of the water content, enzyme concentration and the presence of additives in the yield ester (conversion degree) and the enantioselectivity value (E-value) of selected lipases were carried out. Candida rugosa lipase from commercial sources showed good selectivity than other lipases under tested conditions.

- **Foresti M.L. and Ferreira M.L. in 2006** had reported catalysis of fatty acid by chitosan-immobilized lipases. This study revealed that the best operation conditions for ester synthesis are found at mild temperatures and concluded that immobilization on chitosan allowed easy recovery and reuse of the catalytic material, with no activity decrease.

- **Dong Hwan Lee *et al.,* in 2006** had reported immobilization of lipase produced from *Rhizopus oryzae* on silica gel using a cross-linking method. They found that 80% of the enzymatic activity of the immobilized lipase was retained even after 20 consecutive uses of this preparation.

- **Tianqi Wang *et al*., in 2006** had performed immobilization of *Candida* sp. lipase on epoxy activated (1→3)-α-D-Glucan isolated from *Penicillium chrysongenum*. He found that the activity yield was 83.8% under optimum conditions.

- **Keehoon Won *et al.,* in 2006** had reported lipase catalyzed enantioselective esterification of racemic ibuprofen coupled with prevaporation. This was thought to be due to the selective removal of excess water by pervaporation, leading to the reduction of undesirable effects on the enzymatic reaction.

- **Vimla Minovska *et al*., in 2005** had studied lipase immobilized by different techniques on various support materials applied in oil hydrolysis. Among numerous support materials, Amberlite IRC-50 was stable because this immobilizate distinguished itself not only with acceptable operational stability but also with good storage stability.

- **Hongwei Y *et al*., in 2005** reported kinetic resolution of ibuprofen catalyzed by *Candida rugosa* lipase in ionic liquids**.** The esterification with 1-propanol was conducted in seven ionic liquids. In case of [BMIM] PF6, the enantioselectivity was found almost twice that of isooctane.

- **Amir Kabiri Badr in 2005** studied on enzymatic synthesis of natural ethyl acetate in non-conventional media. He reported that semi-pilot scale enzymatic esterification of ethanol and acetic acid with continuous water removal produced a natural flavour compound.

- **Asadulla Khan in 2004** worked on lipase catalyzed enantioselective esterification of flurbiprofen. In his work, production of S-enantiomer ester prodrug for flurbiprofen via esterification reaction using *Candida cylindraceae* lipase in organic solvent was carried out.

- **Pereira.E.B. *et al.*, in 2003** had reported Immobilization and catalytic properties of lipase on chitosan for hydrolysis and esterification reactions. He used this immobilized preparation for direct esterification of a large range of carboxylic acids with a variety of alcohols.

- **Amresh kumar in 2003** carried out lipase catalyzed enantioselective esterification of ibuprofen. The reaction was carried out with *Candida cylindraceae* and *Candida rugosa* lipase in isooctane as solvent and amyl alcohol as primary alcohol.

- **Yuko Ikeda *et al.,* in 2002** had performed enantioselective esterification of racemic ibuprofen in isooctane by immobilized lipase on cellulose acetate-titanium iso-propoxide gel fiber. He reported that the fiber-immobilised lipase almost retained its initial activity and enantioselectivity at 30^0C after 15 repeated 20 h reactions.

- **Ehab Taqieddin *et al.,* in 2002** had reported perm-selective chitosan alginate hybrid microcapsules for enzyme immobilization technology.

- **D. Magnin *et al.*, in 2001** had reported activities in aqueous and organic media and lipase localization of lipase immobilized into porous chitosan beads. In this study he has compared the properties of free and immobilized lipases and concluded that immobilization of lipases in chitoxan enhances their lypolytic activity in aqueous medium and shows significant activity in organic solvents.

- **Banu in 2001** carried out kinetic studies of *Candida rugosa* lipase immobilized on hydrophobic and hydrophilic supports. He showed that affinity of lipase towards

hydrophobic support is higher than that of hydrophilic support.

- **Bhanadarkar S.V and Stephen H. Neau in 2000** had reported the influences of water activity and solvent hydrophobicity on the kinetic resolution of lipase catalysed esterification of flurbiprofen with n-butanol.

- **Benjamin S *et al.*, in 1998** reviewed use of *Candida rugosa* lipase in molecular biology and biotechnology and concluded that they have more range of applications than any other biocatalyst.

- Applications of immobilized lipases to transesterification and esterification reactions in non – aqueous systems were studied by **Mustranta A *et.al* in 1993**.

- **Mustranta A in 1992** had studied the resolution of (R, S)-ibuprofen enantiomers by esterification in different organic solvents using *Candida cylindraceae* lipase. This enzyme preparation had high enantiospecificity for S (+)-ibuprofen in the esterification reaction of racemic ibuprofen with primary alcohols. The esterification yields of secondary alcohols were much lower than those of primary alcohols.

- **Schandl A and Pittner F in 1984** had reported the role of Na^{+} and Ca^{2+} ions on the action of pancreatic lipase studied with the help of immobilisation techniques.By this method it is possible to distinguish between the action of these ions on the lipase molecule itself and their action on the substrate or product.
- **Kaiser D.C. *et al.*, in 1976** had reported the isomeric inversion of ibuprofen R-enantiomer in humans. The resolution of diastereomeric amides formed by reaction with S (-) α-methylbenzylamine was achieved by GLC.

MATERIALS AND METHODS

MATERIALS

Lipase isolated from *Candida rugosa* (819 Units/mg enzyme) was purchased from Sigma Chemical Co. and used.

CHEMICALS

Anhydrous acetic acid	-	S. D. Fine Chem. Ltd., Mumbai.
Amyl alcohol	-	Qualigens Fine Chemicals, Mumbai.
Bovine serum albumin	-	Hi Media Pvt. Ltd, Mumbai
Chitosan	-	Cochin Fisheries
Calcium chloride	-	S. D. Fine Chem. Ltd., Mumbai.
Ethyl acetate	-	Qualigens Fine Chemicals, Mumbai.
Glutaraldehyde	-	Kemphasol
Isooctane	-	Qualigens Fine Chemicals, Mumbai.
n-Hexane	-	Qualigens Fine Chemicals, Mumbai.
Oil of Olive	-	Hi Media Pvt. Ltd, Mumbai
Sodium alginate	-	Loba Chemicals, Mumbai.
Sodium di hydrogen phosphate	-	E. Merck (India) Ltd., Mumbai.

TLC Plates
(Pre-coated Silica gel 60 F_{254}) - S. D. Fine Chem. Ltd., Mumbai.

Tris hydrochloride - Hi Media Pvt. Ltd, Mumbai.

INSTRUMENTS

- CAMAG Linomat V Applicator
- CAMAG Scanner
- Centrifuge
- Digital Balance
- Digital Orbital Shaker
- Glass Wares
- Heating Mantle
- Incubator
- Lab Stirrer
- Magnetic Stirrer
- Micropipettes
- Micro Tips
- Syringe and Needle

METHODS

a) Lowry method of protein estimation

b) Immobilization methods

c) Activity studies of free and immobilized lipases

d) Enantioselective esterification of free and immobilized lipase

a) Protein Assay

Lowry Method

The protein content of lipase was determined spectrophotometrically.

The Lowry procedure has been found to be one of the most reliable and satisfactory method for quantifying soluble proteins. The procedure described here is based on the method adopted by Lowry et al in 1951.

Prepare a standard solution of protein at a concentration of 1mg/ml. Bovine serum albumin is used as standard.

Pipette out 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2ml of standard series in a test tube. Now pipette out different unknown concentration. Make up the volume in all the test tubes. A test tube with 1ml of water is used as the blank. Add 5 ml of Alkaline copper solution containing 2% Na_2CO_3 in 0.1 NaOH to each test tube including blank. Mix well and allow it to stand for 10 minutes. Then add 0.5ml of 0.2 N Folin Phenol reagent. Mix well and incubate at room temperature in dark for 30 minutes. Blue colour develops. Take the reading at 660nm. Draw a standard graph and calculate the amount of protein in the sample.

b) Immobilization method for lipase from *Candida rugosa*

Immobilization of Lipase on Chitosan

Lipase was immobilized by physical adsorption on chitosan following a previously developed methodology with slight modifications. Chitosan *in natura* (2 g) had been previously soaked in hexane under agitation conditions (100 rpm) for 1 hour.

Then, excess hexane was removed followed by the addition of 0.5 grams of powder lipase dissolved in 10 mL of distilled water. The lipase was on the support under agitation for 3 hours at room temperature followed by an additional period of 18 hours under static conditions at 4ºC. The derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with hexane. **E.B.Pereira *et al* 2003).**

Immobilization of lipase on glutaraldehyde pre-treated chitosan

i) Preparation of glutaraldehyde-pretreated chitosans

Glutaraldehyde-modified supports were prepared by suspending 375 mg of chitosan powder in 50 ml of 0.025% or 0.25% (v/v) glutaraldehyde/phosphate buffer pH 7.0 solutions. The suspension was kept under 800 rpm stirring at room temperature for 30 min. After that time the supports were recovered by filtration and washed with distilled water. The modified supports (CHIT/GLU 0.025 and CHIT/GLU 0.25) were stored at 50ºC for 12 h.

ii) Lipase immobilization

The immobilization of lipases was performed at room temperature during 7 h with 350 rpm stirring. Phosphate buffer kept pH at 7, and the ionic strength of the immobilization medium equal to 0.014 M. In the case of CRL (solid powder), 150 mg of these lipases were added to 50 ml of phosphate buffer solutions, and subjected to strong stirring during 30 min in order to solubilize lipase. A filtering step was then performed in order to retain carbohydrates and other insoluble compounds. In the case of CALB (liquid ambar solution), 0.9 ml of the enzyme commercial solution were diluted up to 50 ml with phosphate buffer. Each lipase solution was then contacted with 375mg of chitosan powder and glutaraldehyde-pretreated chitosans (room temperature, 350 rpm). After the desired contact time (7–8 h) solids were recovered by filtration, washed with distilled water and dried at 50ºC for 12h **(M.L. Foresti and M.L. Ferreira in 2006).**

Immobilization of Lipase by Gel entrapment

The lipase powder was dissolved in Tris–HCl buffer (50 mM, pH 7.3) to obtain a 4 % w/v solution. This solution was added to sodium alginate to yield either a 1.5 % w/v or a 2.5 % w/v mixture which was left to deaerate over night at 25 °C. By dropping the lipase–alginate mixture through a syringe into an aqueous solution of $CaCl_2$ (0.1 M), gel beads of about 3-mm diameter were formed within 3 to 5 min. The beads were kept in $CaCl_2$ solution until used.

c) Activity Studies of Free and Immobilized Lipases

Determination of Lipase Activity

The activity of the free and immobilized lipase was determined by hydrolyzing olive oil at room temperature and at pH 7.0. In a 25 ml beaker, 1 ml 1.0 N NaCl and 50 mM $CaCl_2$ solution and 4 ml of 25 mM phosphate buffer at pH 7.0 placed unless otherwise stated. Then, 1 ml of free or immobilized enzyme solution in varying concentrations was added. After the equilibration of the pH value, 5 ml of olive oil was added. The pH was maintained by the addition of 50 mM NaOH solution (Yang and Chen, 1994).

Activity (U/mg solid) was calculated by the following formula:

$$A = \frac{\text{Normality of NaOH} * 1000 * \text{Amount of NaOH expended (ml)}}{\text{Amount of Enzyme (mg)}}$$

Effect of pH

Effect of pH on free and immobilized enzymes was investigated with the incubation of enzyme solutions at different pH such as 5, 5.5, 6, 6.5, 7.0, 7.5 and 8 for 30 minutes in assay medium and then the activity was determined.

Effect of Temperature

Effect of temperature on free and immobilized enzymes was investigated with the incubation of enzyme solutions at different temperatures such as 20, 25, 30, 35, 37, 40, 50 and 60^0C for 30 minutes in assay medium and then the activity was determined.

d) Enantioselective Esterification of Free and Immobilized Lipases

Esterification method

Biocatalytic esterification of ibuprofen racemate was carried out using Free *Candida rugosa* lipase and Immobilized *Candida rugosa* lipase. In this esterification reaction isooctane and amyl alcohol were used as solvent and primary alcohol respectively.

The selection of suitable solvent and alcohol were optimized earlier in the work done by **Asadulla Khan in 2004.**

Reaction conditions for esterification of ibuprofen (Tsai SW *et al* 1997)

With free *Candida rugosa* lipase:

The reaction mixture consisted of 40ml of the solvent(isooctane), 300mg of free *Candida rugosa* lipase, 500mg each of $Na_2HPO_4.7H_2O$ and $Na_2HPO_4.2H_2O$ (to control water activity), 0.6mM of racemic ibuprofen and 12mM of primary alcohol(amyl alcohol). The reaction mixture was allowed to equilibrate at the desired water activity by shaking in a constant temperature shaker bath at 30^oC for 24 hours. 12mM of amyl alcohol was added to start the reaction and the process was continued for 192 hours with continuous shaking (150-200 rpm).

Samples were withdrawn at regular intervals (0, 24, 48, 96, 168 and 192 hours) and monitored for the esterification reaction by HPTLC method.

With immobilized *Candida rugosa* lipase:

The reaction mixture consisted of immobilized beads prepared earlier containing *Candida rugosa* lipase enzyme equivalent to 300mg of free *Candida rugosa.*

All the other conditions were similar to that used for free lipase containing reaction mixture process, described.

Blank preparation:

Blank was prepared for each set of reaction. Blank contained only the solvent without corresponding alcohol, without ibuprofen and without lipase (free and immobilized) respectively. Samples were withdrawn at regular time intervals as done in the test and monitored using HPTLC, to confirm any interference in the study.

High Performance Thin Layer Chromatography

The HPTLC system consisted of Linomat V applicator and CAMAG scanner. Analysis of ibuprofen at the start of the reaction ('0' hrs) and esterified ibuprofen samples (24, 48, 96, 168 and 192 hrs) was performed by application of spots on the pre-coated TLC plate. 5µl of the sample was applied onto a pre-coated TLC plate and mobile phase of n-hexane-ethyl acetate-anhydrous acetic acid (75:25:2) was used for the development of the TLC. After development, the spots were scanned by the densitometric CAMAG scanner at 254nm. Samples were prepared by centrifuging the reaction mixture for 10 minutes at 5000 rpm to remove the enzyme. 100µl of the clear supernatant organic layer was collected and 5µl was applied onto the TLC plates.

Conditions of HPTLC

Mobile phase	:	n-hexane-ethyl acetate-anhydrous acetic acid (75:25:2)
Sample injected	:	5µl
UV detector	:	254nm
Injector	:	Linomat V injector
Scanner	:	CAMAG scanner

RESULTS

Immobilization of lipase

Immobilized beads of *Candida rugosa* lipase was prepared by using various supports.

1. Entrapment into calcium alginate beads
2. Covalent bonding onto glutaraldehyde pretreated chitosan
3. Physical adsorption on chitosan

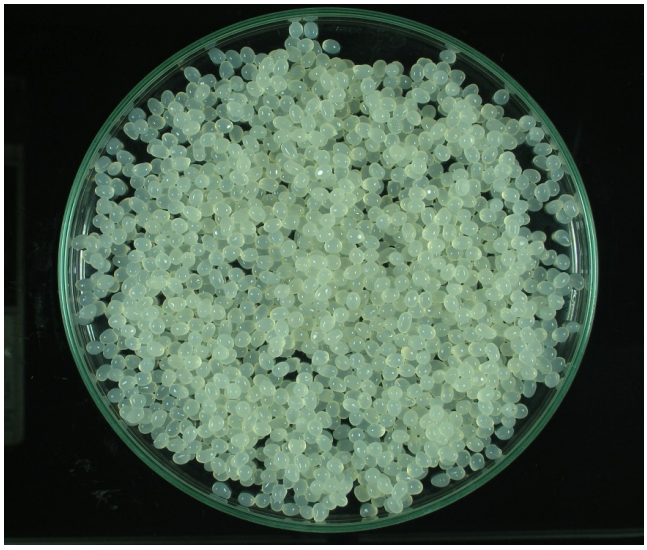


Figure 5: Immobilized beads of calcium alginate

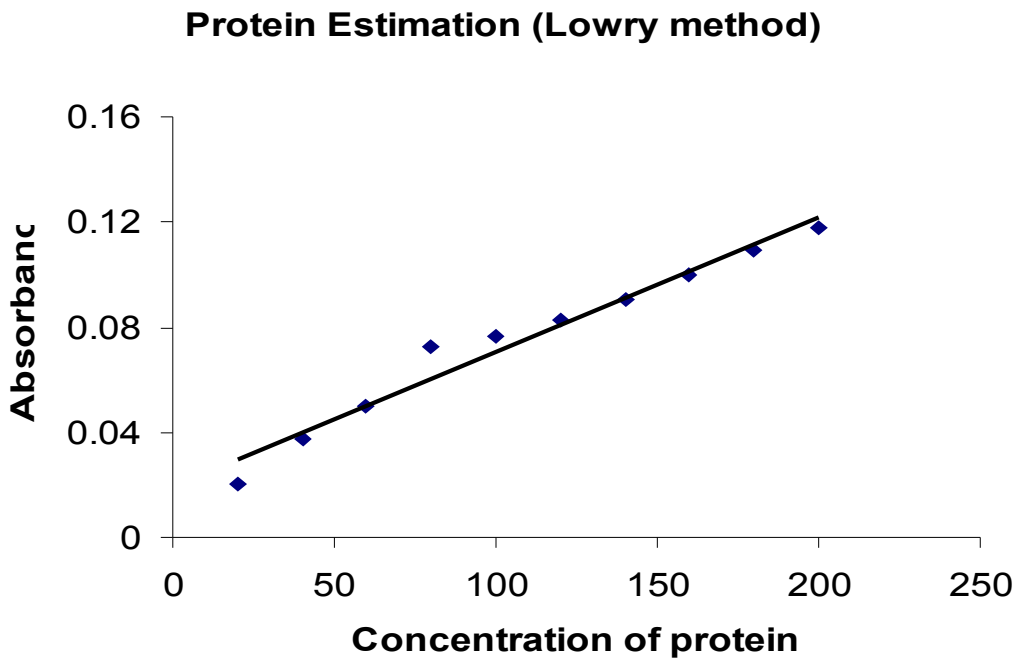
Figure 6: Lipase immobilized on glutaraldehyde pretreated chitosan

Figure 7: Lipase immobilized on chitosan

Protein assay

Table: 2 Preparation of standard curve of albumin

Figure 8: Standard curve of albumin



Absorbance of enzyme solution = 0.072

Concentration of protein present in the enzyme solution = 80µg/ml

pH studies:

Table: 3 Estimation of optimum pH for free lipase

Optimum pH – 7

Figure 9: Effect of pH on free lipase

Table: 4 Estimation of optimum pH for calcium alginate beads

Optimum pH – 7

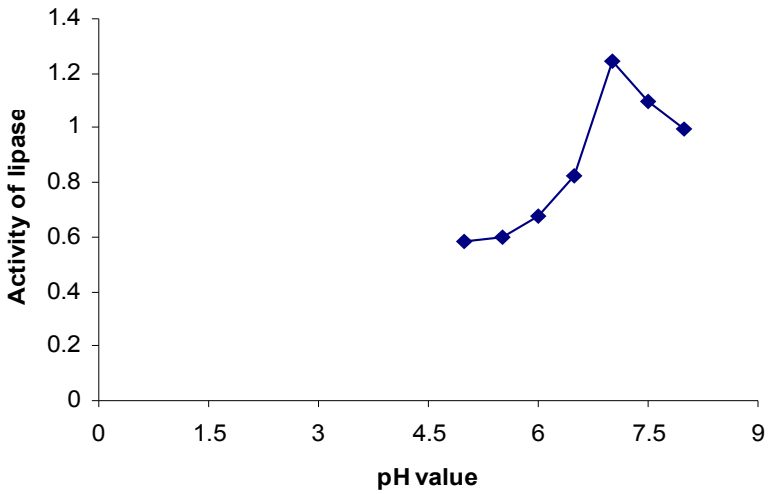


Figure 10: Effect of pH on immobilized calcium alginate beads

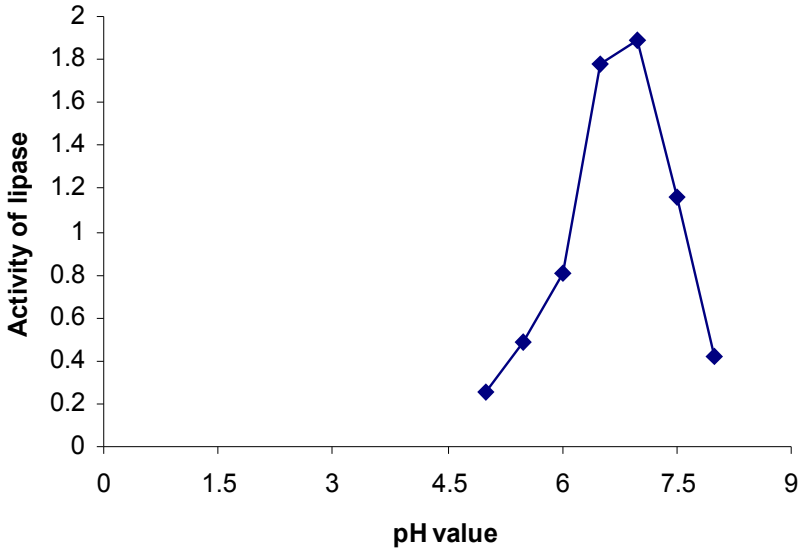


Table: 5 Estimation of optimum pH for lipase immobilized on glutaraldehyde pretreated chitosan

Optimum pH – 6.5

Figure 11: Effect of pH on lipase immobilized on glutaraldehyde pretreated chitosan

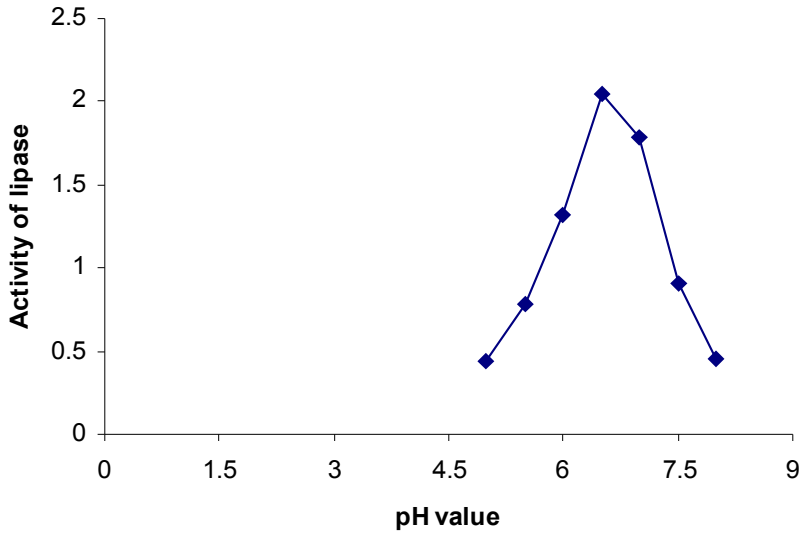
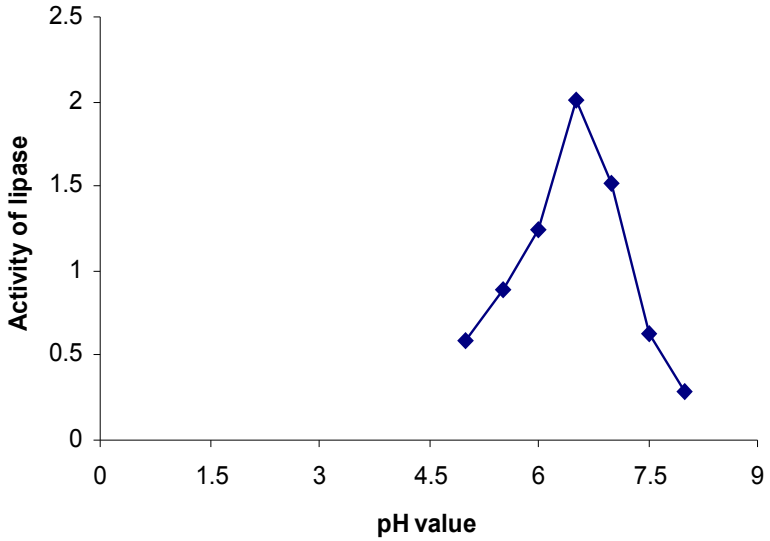


Table: 6 Estimation of optimum pH for lipase immobilized on chitosan

Optimum pH – 6.5

Figure 12:
Effect of
pH on lipase immobilized on chitosan



Temperature studies:

Table: 7 Estimation of optimum temperature for free lipase

Optimum

Temperature- 40^0 C

Figure 13: Effect of Temperature on free lipase

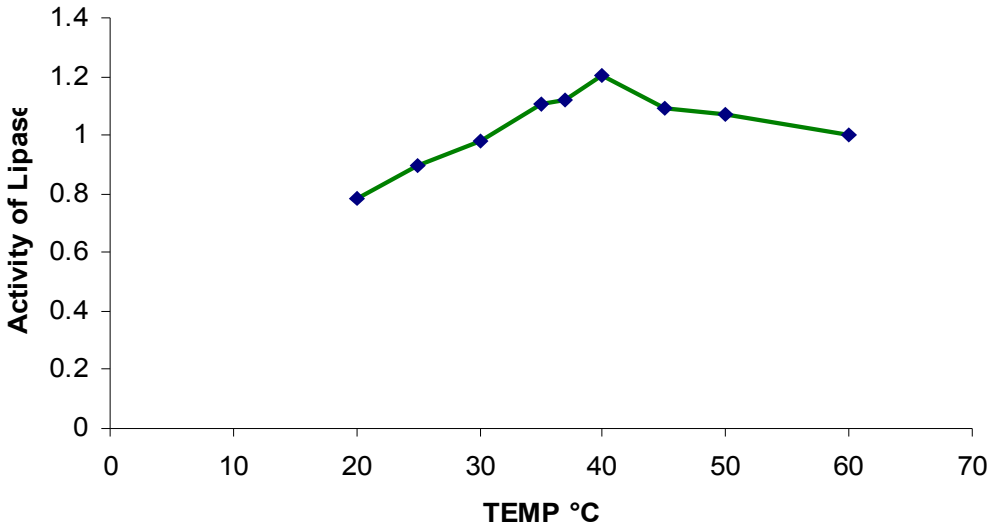


Table: 8 Estimation of optimum temperature for calcium alginate beads

Optimum

Temperature- 37^0 C

Figure 14: Effect of Temperature on immobilized calcium alginate beads

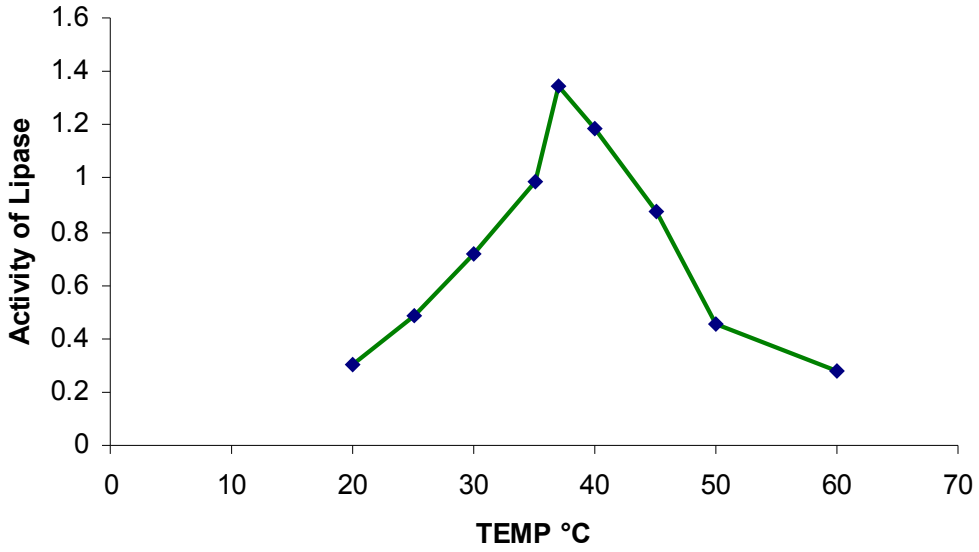


Table: 9 Estimation of optimum temperature for lipase immobilized on glutaraldehyde pretreated chitosan

Optimum
Temperature- 45^0 C

Figure 15: Effect of Temperature on lipase immobilized on glutaraldehyde pretreated chitosan

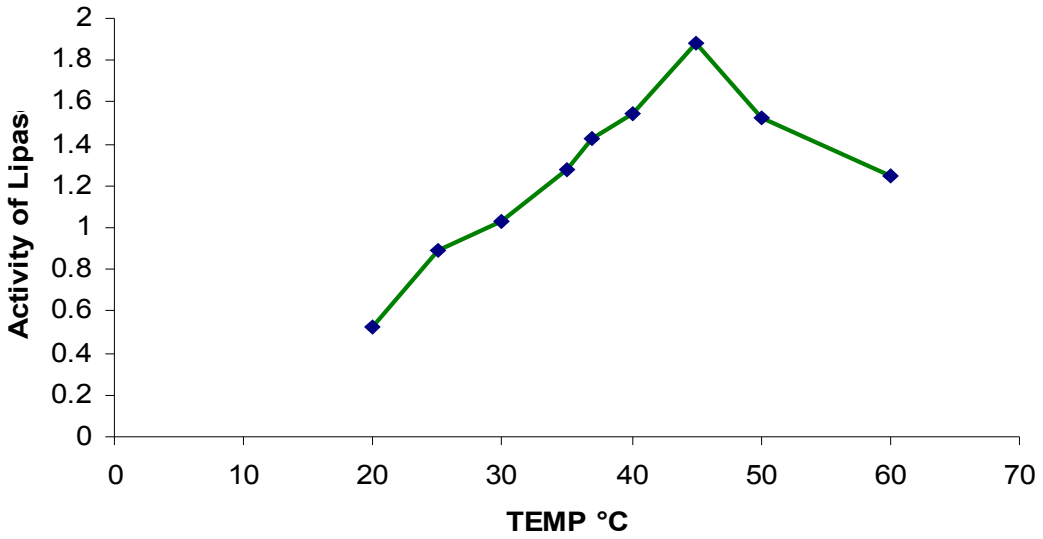
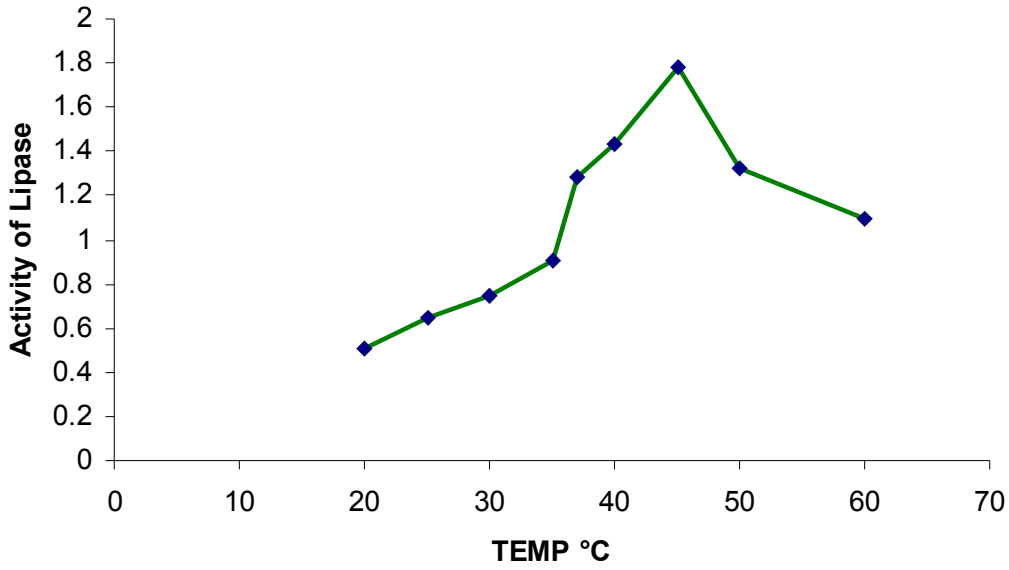


Table: 10 Estimation of optimum temperature for lipase immobilized on chitosan

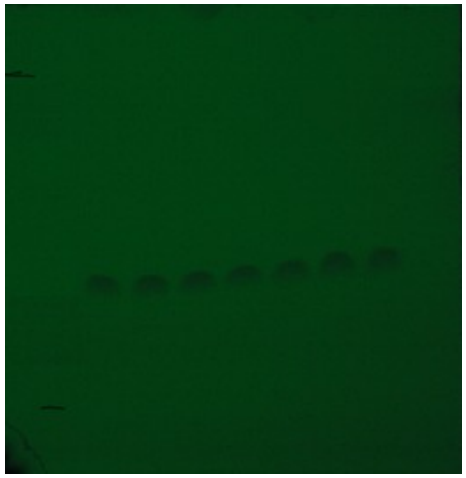
Optimum

Temperature- 45^0 C

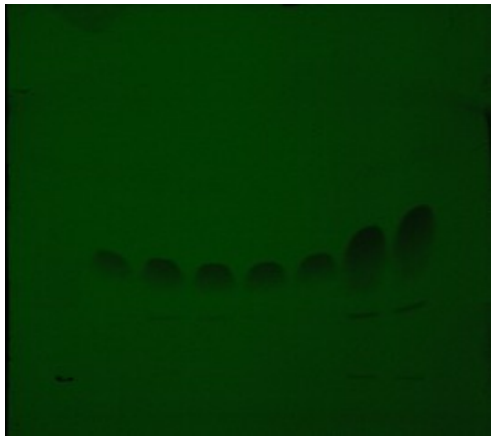
Figure 16: Effect of Temperature on lipase immobilized on chitosan



HPTLC Results



HPTLC for free lipase



HPTLC for calcium alginate beads

HPTLC for lipase immobilized on pretreated chitosan chitosan

HPTLC for lipase immobilized on glutaraldehyde

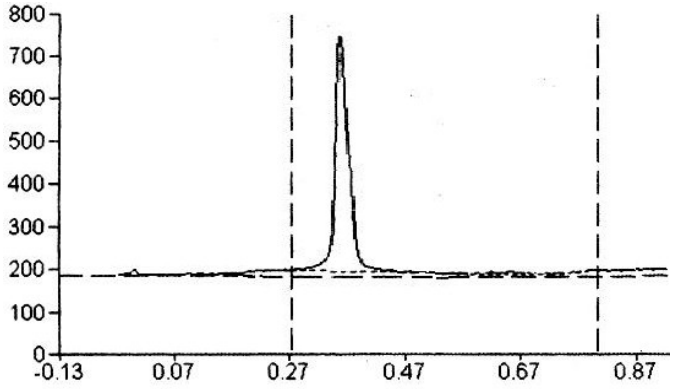
HPTLC results of esterification of ibuprofen with amyl alcohol using free *Candida rugosa* lipase

The HPTLC chromatogram of sample at '0' time showed one peak with R_f value of 0.35 corresponding to free ibuprofen, 24 hours sample showed two peaks with R_f value 0.35 and 0.68 respectively where the first peak corresponds to the unreacted ibuprofen and second peak corresponds to amyl ester of ibuprofen. Similarly 48hrs, 96hrs, 168hrs and 192hrs samples have shown two peaks corresponding to the acid and ester respectively.

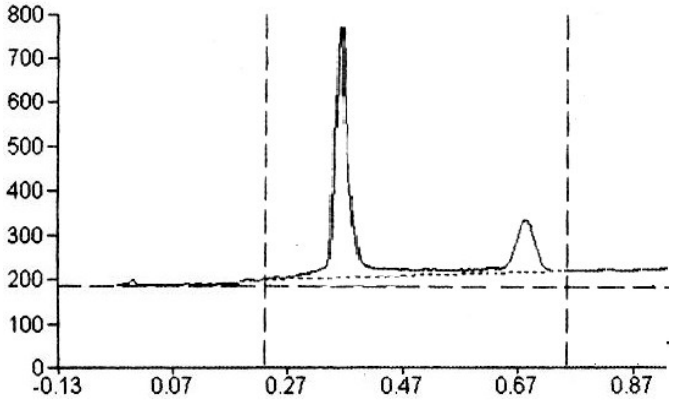
Table: 11

Sample time	Peak	R_f value	Peak area	% of free Ibuprofen
0 hr	Peak 1 Peak 2	0.35 -	17760.5 -	100% -
24 hr	Peak 1 Peak 2	0.35 0.68	16545.7 7132	93.16%
48 hr	Peak 1 Peak 2	0.35 0.65	16147.8 8942.2	90.92%
96 hr	Peak 1 Peak 2	0.32 0.62	15334.4 9842	86.34%
168 hr	Peak 1 Peak 2	0.3 0.62	14048.6 13712	79.10%
192 hr	Peak 1 Peak 2	0.30 0.62	13711.1 14024	77.20%

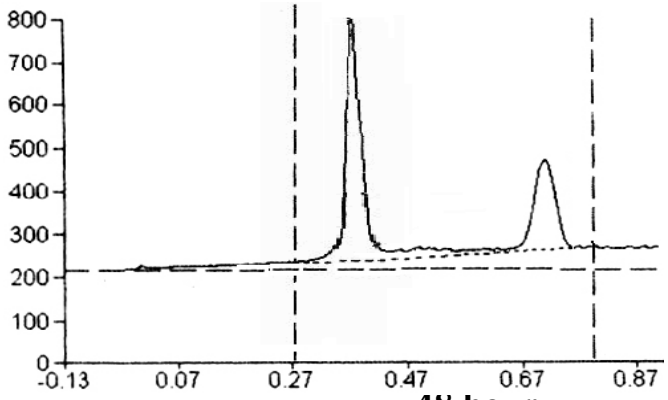
Figure: 18 HPTLC results of the esterification of ibuprofen using free *Candida rugosa* lipase



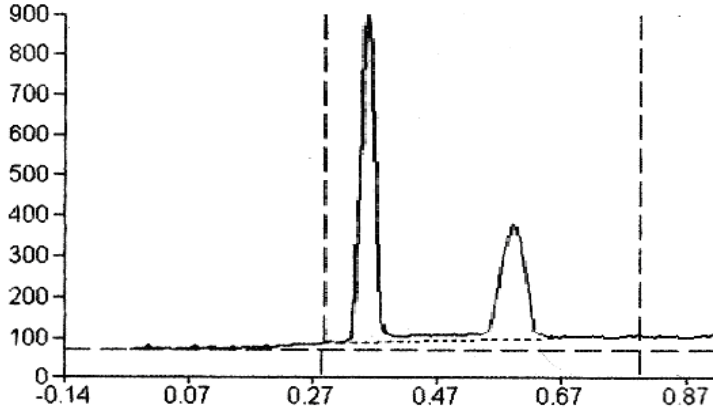
0 hour



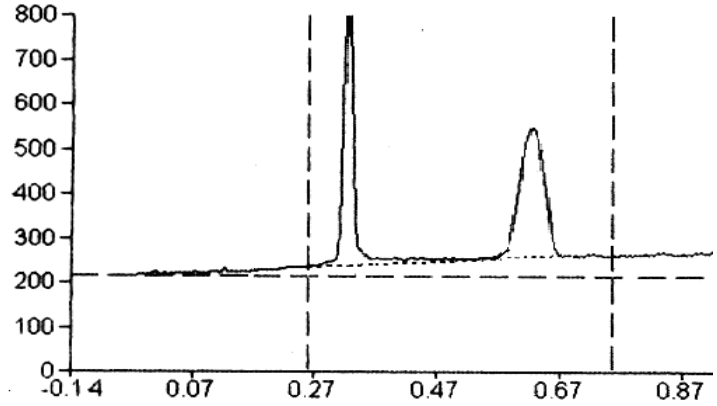
24 hour



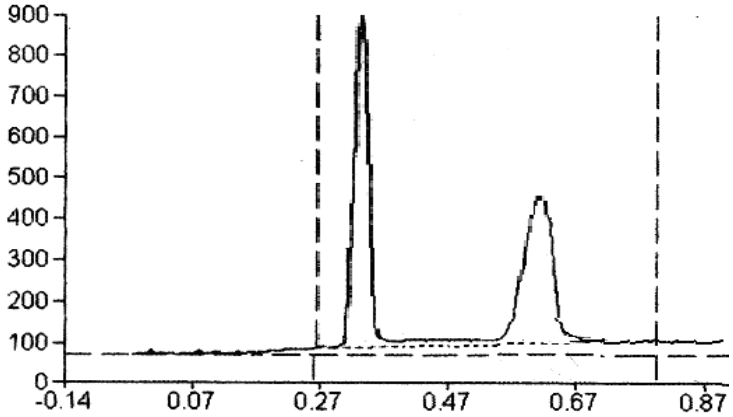
48 hour



96 hour



168 hour



192 hour

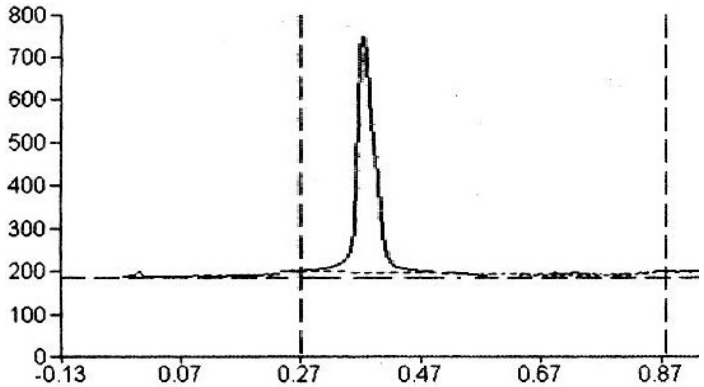
HPTLC results of esterification of ibuprofen with amyl alcohol using immobilized *Candida rugosa* lipase (calcium alginate)

The HPTLC chromatogram of sample at '0' time showed one peak with R_f value of 0.38 corresponding to free ibuprofen, 24 hours sample showed two peaks with R_f value 0.38 and 0.70 respectively where the first peak corresponds to the unreacted ibuprofen and second peak corresponds to amyl ester of ibuprofen. Similarly 48hrs, 96hrs, 168hrs and 192hrs samples have shown two peaks corresponding to the acid and ester respectively.

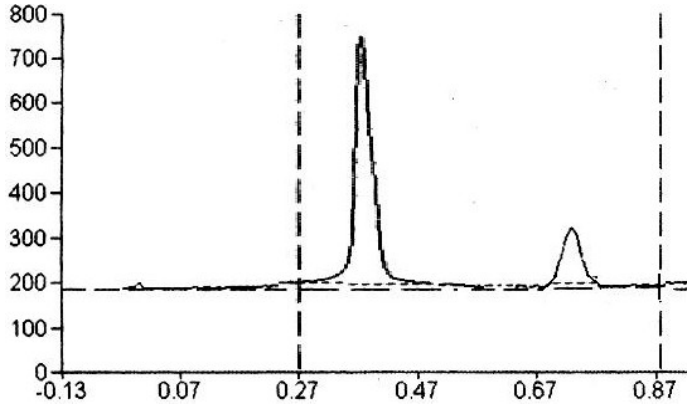
Table: 12

Sample time	Peak	R_f value	Peak area	% of free Ibuprofen
0 hr	Peak 1 Peak 2	0.38 -	16152 -	100% -
24 hr	Peak 1 Peak 2	0.38 0.70	15199 1542	94%
48 hr	Peak 1 Peak 2	0.36 0.68	14897 3147	92.23%
96 hr	Peak 1 Peak 2	0.36 0.68	14795.2 5019	91.6%
168 hr	Peak 1 Peak 2	0.32 0.69	13777.7 8236	85.3%
192 hr	Peak 1 Peak 2	0.32 0.69	13244.6 11452	82%

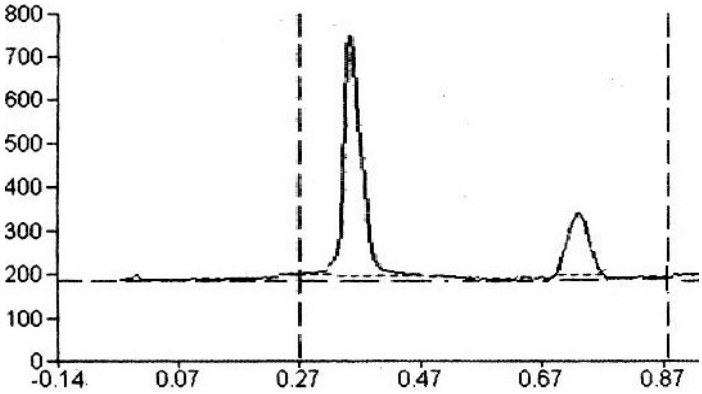
Figure: 19 HPTLC results of esterification of ibuprofen with amyl alcohol using immobilized *Candida rugosa* lipase (calcium alginate)



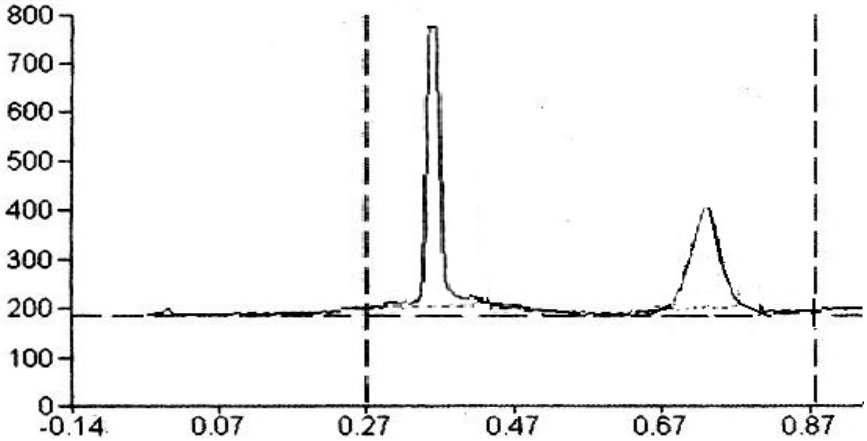
0 hour



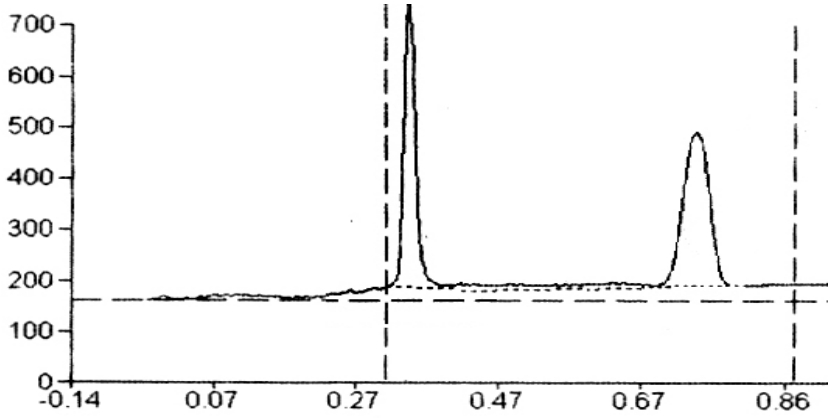
24 hour



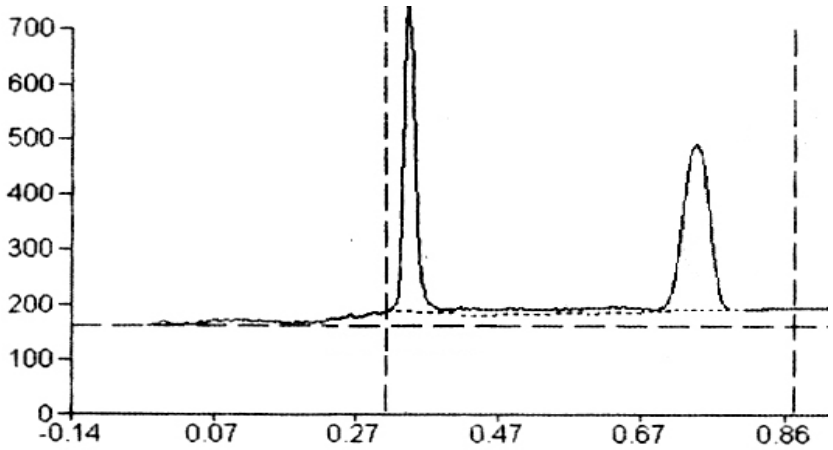
48 hour



96 hour



168 hour



192 hour

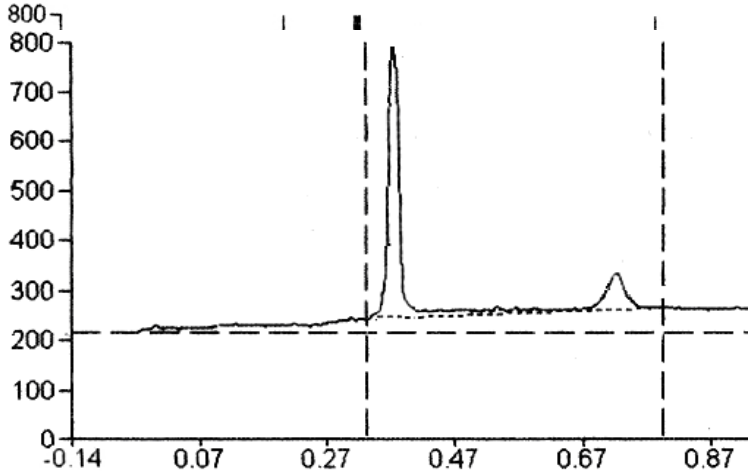
HPTLC results of esterification of ibuprofen with amyl alcohol using immobilized *Candida rugosa* lipase (on glutaraldehyde pretreated chitosan)

The HPTLC chromatogram of sample at '0' time showed one peak with R_f value of 0.36 corresponding to free ibuprofen, 24 hours sample showed two peaks with R_f value 0.36 and 0.69 respectively where the first peak corresponds to the unreacted ibuprofen and second peak corresponds to amyl ester of ibuprofen. Similarly 48hrs, 96hrs, 168hrs and 192hrs samples have shown two peaks corresponding to the acid and ester respectively.

Table: 13

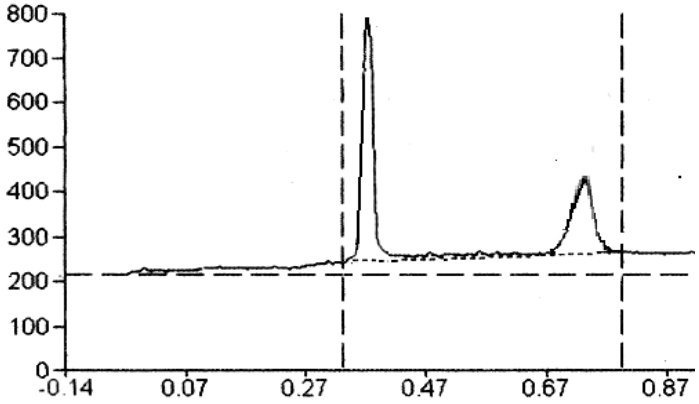
Sample time	Peak	R_f value	Peak area	% of free Ibuprofen
0 hr	Peak 1 Peak 2	0.36 -	14144 -	100% -
24 hr	Peak 1 Peak 2	0.36 0.69	12859.7 4234	90.92%
48 hr	Peak 1 Peak 2	0.31 0.68	12521.6 5624	88.6%
96 hr	Peak 1 Peak 2	0.32 0.67	12135.6 6984	85.8%
168 hr	Peak 1 Peak 2	0.31 0.67	11414.2 8412	80.70%
192 hr	Peak 1 Peak 2	0.31 0.67	11032.3 9988	78.0%

Figure: 20 HPTLC results of the esterification of ibuprofen using *Candida rugosa* lipase immobilized on glutaraldehyde pretreated chitosan

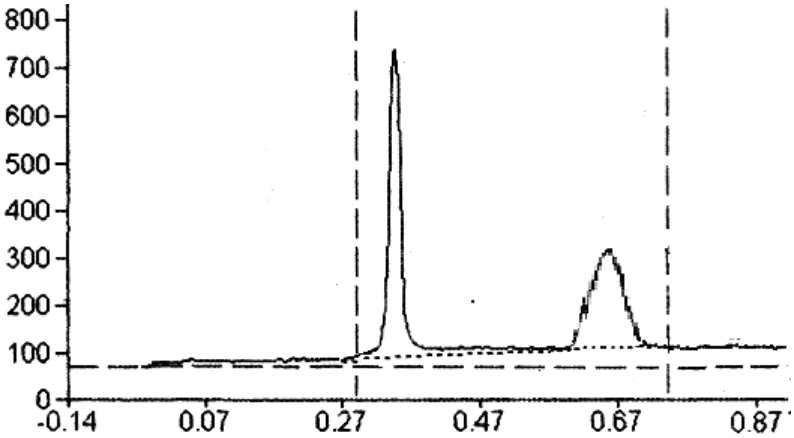


0 hour

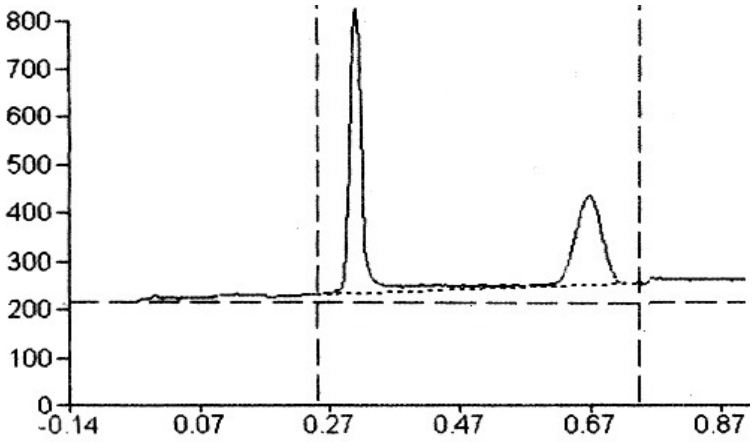
24 hour



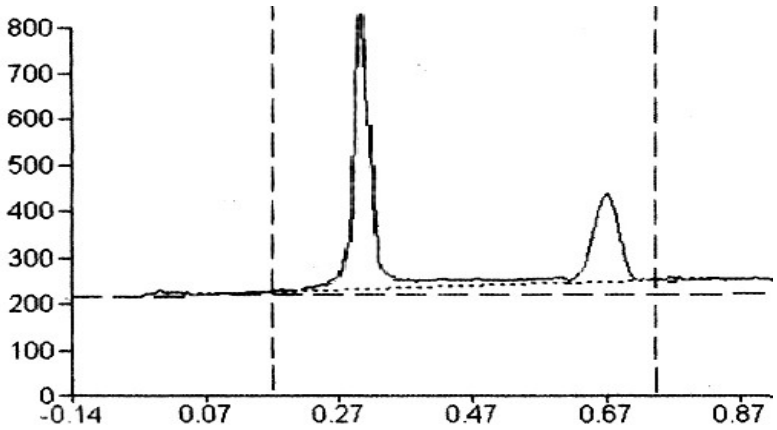
48 hour



96 hour



168 hour



192 hour

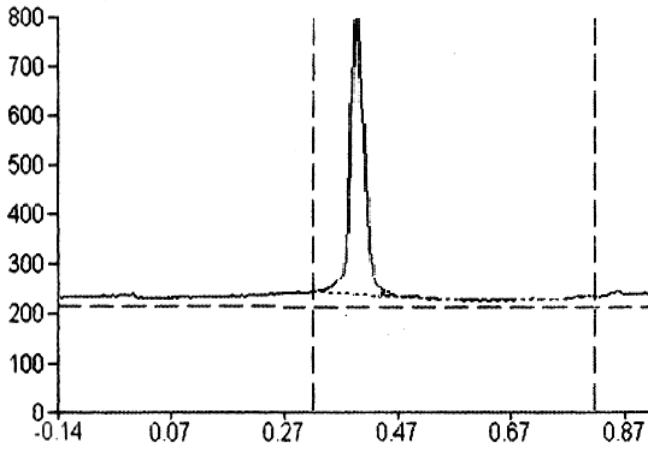
HPTLC results of esterification of ibuprofen with amyl alcohol using immobilized *Candida rugosa* lipase (on chitosan)

The HPTLC chromatogram of sample at '0' time showed one peak with R_f value of 0.39 corresponding to free ibuprofen, 24 hours sample showed two peaks with R_f value 0.39 and 0.72 respectively where the first peak corresponds to the unreacted ibuprofen and second peak corresponds to amyl ester of ibuprofen. Similarly 48hrs, 96hrs, 168hrs and 192hrs samples have shown two peaks corresponding to the acid and ester respectively.

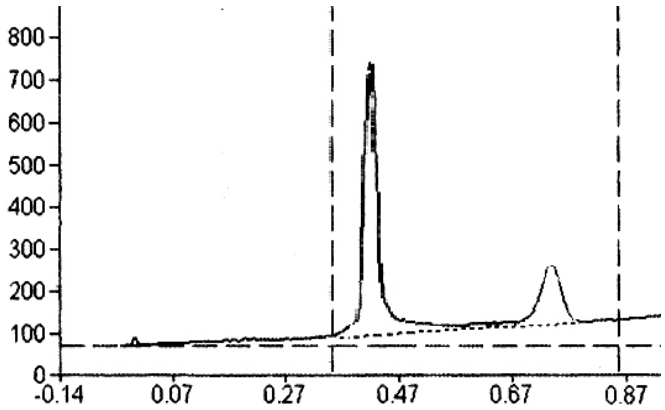
Table: 14

Sample time	Peak	R_f value	Peak area	% of free Ibuprofen
0 hr	Peak 1 Peak 2	0.39 -	13392.4 -	100% -
24 hr	Peak 1 Peak 2	0.39 0.72	13026.8 982	97.27%
48 hr	Peak 1 Peak 2	0.34 0.72	11907.2 2917	88.91%
96 hr	Peak 1 Peak 2	0.32 0.65	11644.7 4562	86.95%
168 hr	Peak 1 Peak 2	0.31 0.64	10231.8 7324	76.4%
192 hr	Peak 1 Peak 2	0.31 0.64	10044.3 8461	75.0%

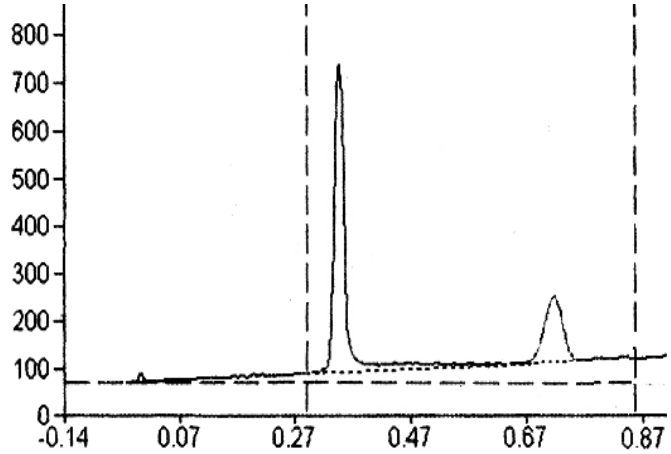
Figure: 21 HPTLC results of the esterification of ibuprofen using *Candida rugosa* lipase immobilized on chitosan



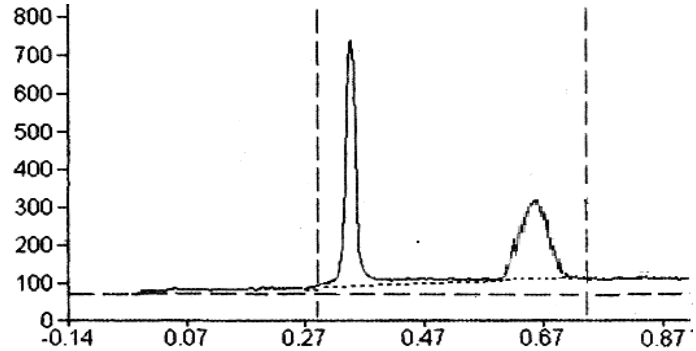
0 hour



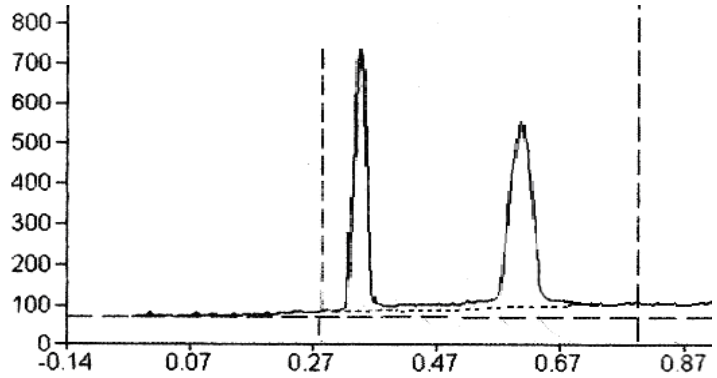
24 hour



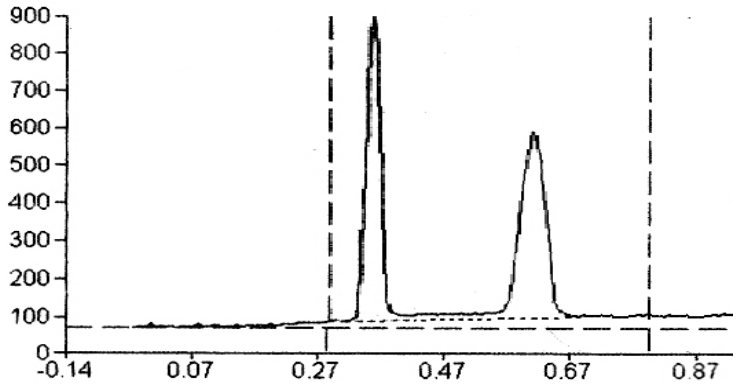
48 hour



96 hour



168 hour



192 hour

CONCLUSION AND EXTENSION OF WORK

Enzymes act on substrates specifically and ultimately cause transformation/ conversion of substrate to products. Enzymes present numerous potential of applications in diversified areas like food technology, environmental and pharmaceutical sectors. Lipase is the most frequently used enzyme because it has an enantioselective reaction property of racemic mixtures. However enzymes are costly because almost all the commercially available lipases are made available from genetically engineered organisms, which in turn make the enzyme expensive. The above drawback can be partly eliminated by immobilization of enzyme onto different supports.

The immobilization of enzymes is a useful tool to meet cost targets and has a number of technological advantages, as for instance, it enables repeated use of the enzyme resulting in significant cost savings. Immobilized enzyme can be easily separated from the reaction liquid and thereby reduces laborious separation steps. Additional benefits arise from stabilization against harsh reaction condition, which are deleterious to soluble enzyme preparation.

Immobilization of enzymes can be carried out by different techniques like adsorption, ionic binding, covalent binding by chemical coupling, cross linking, entrapment and encapsulation.

We have selected the following methods for the advantages mentioned here

1. Calcium alginate beads were prepared by entrapment method which is very simple and inexpensive to perform.
2. Immobilization of lipase on glutaraldehyde pretreated chitosan allows easy recovery and reuse of the catalytic material with no activity decrease. It also gives higher temperature resistance to the enzymes.
3. Physical adsorption advantages like simplicity, little effect on activity of

biocatalyst and possibility of regenerating inactive enzyme by addition of fresh enzyme.

Activity studies were carried on various supports like entrapment into calcium alginate beads, covalent bonding of lipase on glutaraldehyde pretreated chitosan and on chitosan by physical adsorption from which it was seen that lipase immobilized on glutaraldehyde pretreated chitosan by covalent bonding and on chitosan by physical adsorption exhibited maximum activity at pH 6.5 and at 45°C.

Enantioselective esterification of ibuprofen to produce S- enantiomer ester was carried out by enzymatic process using lipase from *Candida rugosa* as the biocatalyst in the presence of amyl alcohol and isooctane as the primary alcohol and solvent respectively.

Enzyme activity and enantioselectivity could be improved by medium engineering, immobilization, optimization of cultural conditions, genetic engineering and chemical modifications.

Esterification reaction has been more effective when surfactant coated lipase was used (Ganesh R *et al,* 2008). The lipase used in that study was porcine pancreatic lipase. The same modification could be tried for *Candida rugosa* that has been used in our work.

Surfactant coating of lipases is known to increase the lipase activity by a mechanism which “opens-up” the lid covering the active site of the enzyme. If a surfactant coated lipase is used in esterification reaction under optimum conditions, the percentage conversion of ester could be enhanced.

Natural supports like kaolin could be tried for immobilization of lipases (Abdul Rahman *et al,* 2005). *Rhizopus oryzae* lipase immobilized on cellulose fibers was used in esterification to synthesize ethyl oleate (Maha Karra-Chaabouni *et al,* 2008).

On similar lines our work on the enantioselective esterification could be extended for increased yields of the eutomer form of Ibuprofen that is the S(+) enantiomeric form of the drug.

Variables like drug selected for conversions, alcohol used for esterification, solvent used, effect of immobilization, water content and other factors could be optimized for increased yield of active enantiomer.

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